REMARKS

In the Final Office Action dated May 23, 2007, claims 1-24 are pending and are under consideration. Claims 1-24 are rejected as allegedly obvious over U.S. Patent No. 4,675,296 to Lehmussaari et al. ("the '296 patent") in view of Marinchenko et al. (*Appl. Biochem. Microbiol.* 15(6): 670-73 (1979)) ("Marinchenko").

Applicant has amended Claim 1 by reciting that the cereal is ungerminated. Support for the term "ungerminated" is found on the bottom of page 4 of the specification. No new matter is introduced by the amendment to Claim 1.

This Response addresses the Examiner's only rejection. Applicant therefore respectfully submits that the present application is in condition for allowance. Favorable consideration of all pending claims is therefore respectfully requested.

The Examiner has maintained the rejection of Claims 1-24 as allegedly obvious over U.S. Patent No. 4,675,296 to Lehmussaari et al. ("the '296 patent") in view of Marinchenko et al. (*Appl. Biochem. Microbiol.* 15(6): 670-73 (1979)).

The '296 patent is directed to a process for preparing a commercial β-amylase product from whole or at least partially dehusked barley grain by extracting the grain with water, which may contain a reducing agent. As described in column 2, lines 30-40 of the '296 patent, the grain surfaces in the barley grain act as a semi-permeable filter, allowing the β-amylase and some low molecular weight substances to pass into the water. In other words, the '296 patent teaches that the grain's surface should be kept intact to maintain the grain surface so that it can act as a filter. Moreover, the '296 patent does not utilize cellulase to extract β-amylase from the barley grain. A review of the '296 patent clearly reveals that the '296 patent does not teach, disclose or suggest or

even recite the use of cellulase in its process for preparing the β-amylase product, a position with which the Examiner agrees.

On the other hand, the present invention recognizes that the use of the enzyme cellulase in a process of extracting β-amylase from cereal grains surprisingly improves the yield of β-amylase and reduces the extraction time. The cereal grains in the present invention can be unhusked, dehusked, milled, ground or polished grains. In contrast to the present invention, the '296 patent specifically teaches that the grain surface layer underneath the husk should be kept intact so that the surface layer can function as a filter to obtain a β-amylase with minor impurities. Since cellulase breaks down the surface structures underneath any husk of a living grain, the use of cellulase in extracting β-amylase from cereal grains, as presently claimed, would contradict the teaching in the '296 patent. In other words, the '296 patent actually teaches away from the use of cellulose, as in the present invention.

In the Final Office Action, the Examiner alleges that the '296 patent discloses extracting β-amylase from ground or otherwise degraded material, such as barley flour. The Examiner refers to Example I of the '296 patent.

However, Applicant observes that the '296 patent obtains a β-amylase extract from whole or partially dehusked grains utilizing only reducing agents which, according to the '296 patent, convert based β-amylase to free amylase without the use of "arduous purification" steps post extraction and without breaking the grain surface. Inasmuch as cellulase breaks down the surface structure underneath any husk of a living grain, (see page 6, lines 9-16 of the instant application), the '296 patent would not utilize cellulase and actually teaches away from the use of cellulase as it breaks the grain's surface. As acknowledged in the '296 patent, the prior art has already disclosed the use of ground or crushed grains for extracting β-amylase.

Example I of the '296 patent merely demonstrates that the use of whole or dehusked barley (without breaking grain surface) can yield 35% of extractable β-amylase, and at the same time the β-amylase extract prepared from whole or dehusked barley contains fewer other grain ingredients than the β-amylase extract prepared from ground and crushed grains.

Thus, Applicant respectfully submits that based on the teaching of the '296 patent, adding cellulase to the barley used in the '296 patent, including the dehusked barley, would break the grain surface structures underneath the husk, thereby destroying the use of the grain surface layers to act as a filter and block other grain ingredients from mixing with the β-amylase extract, as required in the '296 patent. Thus, Applicant respectfully submits that the '296 patent would not have provided any motivation to those skilled in the art to consider using cellulase in the process.

The Examiner has alleged in the Final Action that the secondary reference to Marinchenko provides the motivation to use cellulase. The Examiner contends that Marinchenko teaches an increase in net amylolytic activity by freeing amylases. The Examiner contends that by referencing amylases in the plural, Marinchenko teaches using cellulose to treat barley materials to increase both α and β amylases. The Examiner alleges that the likelihood that β–amylase would also be increased is not excluded.

Marinchenko teaches enhancing amylolytic activities in malt for saccharification of starch used in the brewing industry. It is not used to remove β-amylase from cereal, as in the present application. Thus, Marinchenko is directed to a totally different field of use as the '296 patent and there is no reason to combine its teaching with the '296 patent in the first instance. Moreover, the '296 patent teaches away from combining it with Marinchenko. As indicated hereinabove, the '296 patent teaches that the grain surface layer underneath the husk should be

kept intact so that the surface layers can function as a filter. Thus, it teaches away from the use of a substance that breaks down the surface structure, such as cellulose. Accordingly, since Marinchenko utilizes cellulose, the '296 patent teaches away from combining it with Marinchenko.

Moreover, Applicant respectfully submits that Marinchenko does not distinguish between α and β amylases. It is respectfully submitted that freed α amylase, rather than β amylase, is responsible for the increased activities observed by Marinchenko. In this connection, Applicant respectfully submits an illustration of a mature cereal grain. See Exhibit A. As shown in Exhibit A, α amylase and β amylase are located in totally different parts of the grain in cereal (such as barley and wheat). As further illustrated and described by Exhibit D (*Enzymers and Their Role in Cereal Technology* by J.E. Kruger et al., American Association of Cereal Chemists, Inc., St. Paul, Minnesota, 1987), particularly on pages 97 and 122, α amylase is located in the outer layers, mostly in the pericarp and small amounts are present in the aleurone. In contrast, as further illustrated and described by Exhibit B (*Wheat Chemistry and Technology* by Y. Pomeranz, American Association of Cereal Chemists, Inc., St. Paul, Minnesota, 1978), particularly, on page 462, all the β amylase is located in the inner parts of the grain in the starchy endosperm. Applicant also submits Exhibit C (*Cereal Science and Technology* by G.H. Palmer, Aberdeen University Press, 1989) for clarification of the physical form of the percarp, aleurone and endorperm. See, e.g., page102 of Exhibit C.

Applicant respectfully submits that in the malting process, the amount of α amylase increases significantly (100-1000 times) in the outer (pericarp) layer. In the brewing of malt, the objective is to have as much α amylase available to degrade starch. Therefore, it is advantageous in the malting process to use cellulase to increase the total amount of α amylase by

releasing it from the hemicellulose-cellulose matrix of the pericarp of the cell wall. Marinchenko specifically points out that "the content of free amylases should be promoted by enzymes degrading cell walls." See the Abstract of the Marinchenko reference. Applicant respectfully submits that this statement by Marinchenko clearly refers to α amylase which is present in the cell walls. As mentioned above, β amylase is located in the central starchy endosperm part of the grain and not in the cell walls. Applicant also respectfully submits that in contrast to the Examiner's allegation, it is well known in the art, such as the teaching in Exhibits A-D, that β–amylase is not enhanced in the malting process.

Additionally, Applicant respectfully submits that in a brewing or malting process such as the one described by Marinchenko, it would not be considered advantageous to use cellulase to release β amylase so that it would be more easily available, because β–amylase is not located in the hemicellulose-cellulose matrix of the cell wall at all. Marinchenko uses cellulase to a free α amylase from the cell walls and Marinchenko's target of the cell wall is totally different from the one in the present invention, i.e. the grain surface. In contrast, the present invention is focused on extracting β–amylase located in the central endosperm of the grains and not in the cell walls. Thus in contrast to the allegations by the Office Action, Marinchenko does not relate to the extracting of β–amylase from a cereal grain.

Moreover, in contrast to the teaching of Marinchenko, the present invention uses ungerminated grains (see bottom of page 4 of the specification) in which the amount of α amylase is much lower compared to the α amylase content in the malted grains used by Marinchenko. Applicant submits that there is indeed a great difference between malted grains such as those used by Marinchenko and ungerminated grains such as those used in the present

invention. Thus, Marinchenko does not teach, disclose or suggest the use of ungerminated grains.

In summary, it is respectfully submitted that there is no motivation to combine Marinchenko with the '296 patent in the first instance as the '296 patent teaches away from the use of a substance, like cellulase, that breaks the grain surface. Moreover, the teachings of Marinchenko relate to the use of malt for saccharification of starch for use in the brewing industry. Thus the process disclosed in Marinchenko is totally unrelated to the process described in the '296 patent.

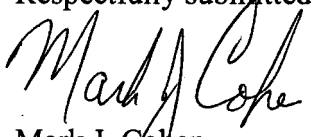
Further, the '296 patent in combination with Marinchenko does not teach or suggest a method for extracting β–amylase from ungerminated cereal, as claimed using cellulase. The '296 patent does not utilize cellulase to obtain β–amylase, but just water and optionally reducing agents in the absence of cellulase, while Marinchenko utilizes cellulase to remove α–amylase without enhancing β–amylase. Thus the combination of the primary and secondary reference do not teach, disclose or suggest the use of cellulase in extracting β–amylase from ungerminated cereal.

Applicant respectfully submits that the secondary reference to Marinchenko does not ameliorate the deficiencies of the primary reference to the '296 patent. The combination of the '296 patent and Marinchenko do not teach the use of cellulase for extracting β–amylase from ungerminated cereal as claimed. Therefore, in view of the foregoing arguments and the amendments to the claims, Applicant respectfully submits that the present invention is not obvious in view of the cited references.

As such, the rejection of Claims 1-24 as allegedly obvious over U.S. Patent No. 4,675,296 to Lehmussaari et al. in view of Marinchenko et al. is overcome, and withdrawal thereof is respectfully requested.

In view of the foregoing amendments and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,



Mark J. Cohen
Registration No. 32,211

Scully, Scott, Murphy & Presser, P.C.
400 Garden City Plaza-STE 300
Garden City, New York 11530
Telephone: 516-742-4343
ZY:ab

Encls.: Exhibits A-D

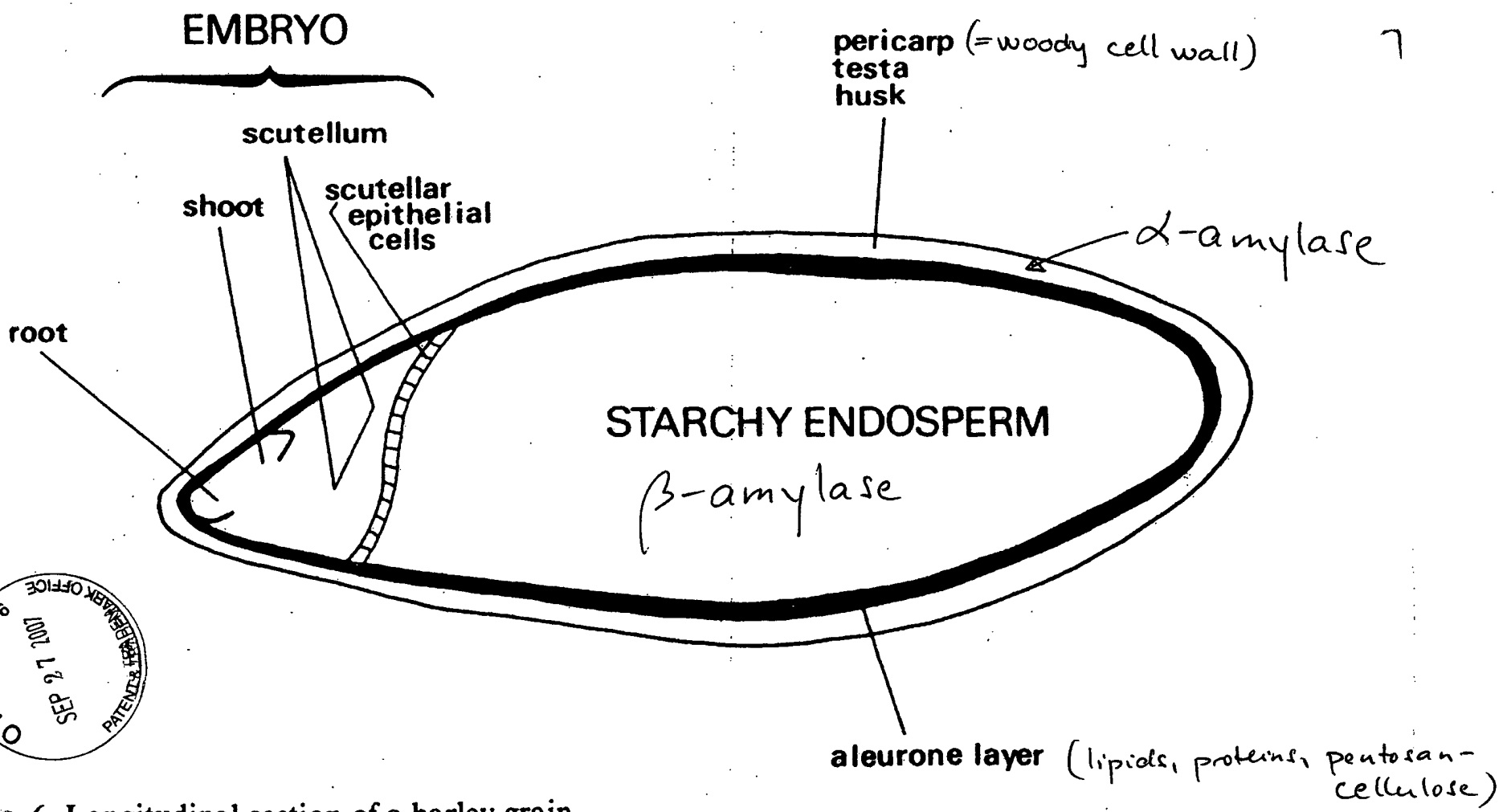


Fig. 6. Longitudinal section of a barley grain.

WHEAT
Chemistry and Technology

Edited by Y. POMERANZ, *National Barley & Malt Laboratory*
USDA, ARS, CRD
Madison, Wisconsin

Published by the

AMERICAN ASSOCIATION OF CEREAL CHEMISTS,
Incorporated
St. Paul, Minnesota

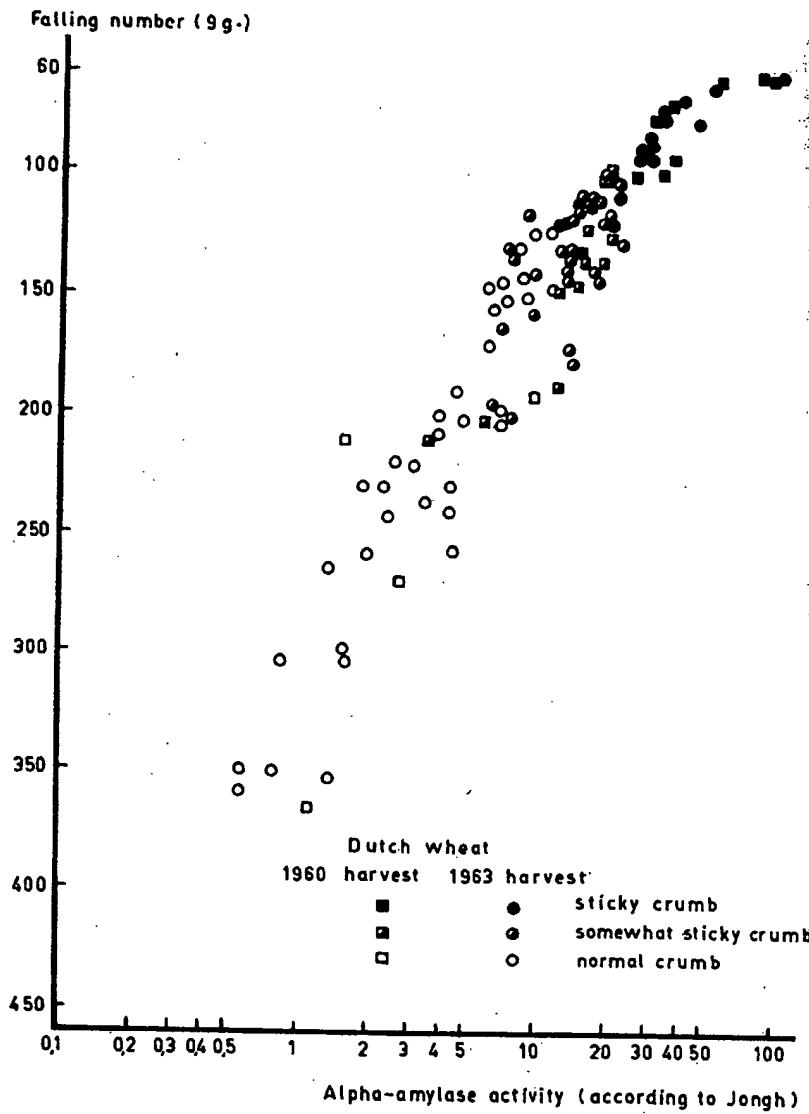


Figure 1. Relation between alpha-amylase activity, falling number value (FN), and bread crumb quality (ref. 148).

dioxide produced by the action of yeast on the fermentable sugars formed during the reaction, or by measuring carbon dioxide volume at constant pressure. This method can be carried out with doughs or sponges approximating commercial formulas, and it is one of the best methods for determining the adequacy of

zyme activity in a flour for practical purposes. At the same time, it is the least liable and least accurate method for determining enzyme activity *per se*, because substrate limitations and limitations in the rate of yeast fermentation. For stance, there is a strongly positive correlation between the amount of damaged arch granules and the sixth-hour gassing-power values (155).

B. Development of Wheat Amylases during Ripening and Germination

Throughout growth and development the amount of alpha-amylase per kernel Baart wheat was about the same (175). This means that the alpha-amylase ontent per g. of dry weight decreases rapidly. However, since the alpha-amylase ontent of ungerminated wheat is quite low, the accuracy of present analytical ethods limits the conclusions which can be drawn. Most authors have, therefore, oncentrated their research on beta-amylase or have restricted their assays to a etermination of total saccharifying power by the Lintner method. This is also true or work on the distribution of the two amylases within the tissue of the grain.

The total amount of beta-amylase increases rapidly throughout growth, and for hatcher wheat it increases roughly in proportion to the dry weight of the kernel, shown in Table II. The proportion of beta-amylase that can be extracted with ater, with salt solution, or after proteolysis with a papain solution shifted hroughout the period of growth. This has led to a distinction between "free" eta-amylase, i.e. beta-amylase that can be extracted with water, and "bound" eta-amylase, which can be extracted after treatment with papain. It is apparent hat bound beta-amylase increases rapidly throughout the growth period, but it is ot clear whether the effect is due to insolubilization of beta-amylase or whether he enzyme is laid down in a different (insoluble) form during the later stages of rowth. For barley, Pollock and Pool have found that bound or "latent" amylase as properties identical with those of the free beta-amylase (151).

Varietal and environmental differences have been investigated in relation to lpha- and beta-amylase activity of ungerminated and germinated wheat and barley. ince varietal and environmental differences were slight in relation to differences

TABLE II

Change in content and solubility of beta-amylase in Thatcher wheat during growth[a]

rowth Stage	Beta-Amylase per g. Dry Weight units	Beta-Amylase Soluble in Water %
arly milk	121	100
ate milk	115	66
oft dough	120	35
tiff dough	127	28
ipe	130	25

[a] ee ref. 175.

TABLE III
Effect of germination on alpha- and beta-amylase activity of wheat and barley[a]

Amylase	Barley		Hard Red Winter Wheat	
	Total	Free	Total	Free
Beta-amylase units				
Ungerminated	28	10 (1 hr.)[b]	23	7 (1 hr.)
Germinated	28	19 (1 hr.)	22	18 (15 min.)
Alpha-amylase units				
Ungerminated	0.04		0.04	
Germinated	54	53 (1 hr.)	201	183 (15 min.)

[a]Data combined from Kneen and Hads (103). (Methods of determination as given in reference.)
[b]Data in parentheses are extraction times.

between wheat and barley, the data (103) could be combined in one table for the sake of simplicity (Table III). It should be emphasized that environmental conditions and germination procedures were not uniform for this set of samples.

The effect of malting conditions and of wheat class on the amylase activity of wheat malts has been studied (52). Free and total alpha-amylase increases in parallel fashion with increases in germination time or germination moisture. Total beta-amylase was almost constant throughout germination. There is a marked increase in the free portion of beta-amylase at the end of the germination period. The results with malts of four wheat classes are shown in Table IV.

Studies with more recently approved wheat varieties have confirmed an optimum steep moisture of 42% and an optimum germination temperature of 20°C. (45). Alpha-amylase values of several wheat classes were as follows: for five soft winter wheats, 360 to 430 units per g.; for five HRW wheats, 360 to 415 units per g.; for two HRS wheats, 340 and 375 units per g.; and for three durum wheats, 360 to 390 units per g. (46). These values are much higher than those shown in Table IV. The longer germination time used in these experiments (12 days vs. 6 days) explains, at least in part, the higher activities reported. The use of potassium

TABLE IV
Amylase activity of wheat malts[a]

Wheat Class	Alpha-Amylase Activity per Gram[b]	Beta-Amylase Activity per Gram[b]
Soft white	79.6 (97%)	15.2 (63%)
Soft red winter	74.4 (97%)	22.1 (58%)
Hard red spring	71.3 (91%)	22.4 (45%)
Hard red winter	62.9 (90%)	19.2 (43%)

[a]See ref. 52.
[b]Activity given is total activity. "Free" activity is given in parentheses.

gibberellate at levels of 0.001 to 0.005% in the steep water led to higher alpha- and beta-amylase activities in wheat malts (44).

C. Distribution of Amylases in the Wheat Kernel

Wheat grains were sliced into halves perpendicular to the long axis of the grain (174). The top halves contained roughly the same amount of alpha- or beta-amylase as the bottom halves; this merely permits the conclusion that amylases are not concentrated in the germ (144). Similarly, an investigation of different milling fractions yielded data which do not permit definite conclusions as to the exact location of the amylases of the grain. The amylase concentrations that have been reported (174) are given in Table V.

To determine the location with accuracy, it was necessary to slice the grain with a microtome and to determine amylase activity in slices 25 μ thick. This permitted Mogel to plot the distribution of beta-amylase as shown in Figure 2 (38,39). It was found that the amount of amylase in the inner portion of the endosperm is small and that it increases toward the outer portions, so that the first starch-containing cell layers adjoining the aleurone cells are particularly rich in amylase. The aleurone cells and the bran contain no beta-amylase. In the germ, amylase is located in the scutellum.

D. Amylase of Other Cereal Grains in Relation to Wheat

It is difficult to find extensive data on the alpha- and beta-amylase content of cereals which permit a valid comparison between different cereals, because most authors have dealt with a small number of cereals and, within each class, with a small number of varieties. A combination of their results is rarely possible, because their methods for determining enzyme activity vary. The widest survey in this field was made by Davidson, whose results are shown in Table VI (33). Unfortunately, enzyme activity has been determined by the Lintner method, which does not distinguish between alpha- and beta-amylase activity; consequently, results have to be reported as total saccharifying activity.

Table VI shows that ungerminated barley, wheat, and rye have high activities and that germination increases saccharifying power, particularly in the fraction

TABLE V
Amylase activity of mill fractions[a]

Mill Fraction	Beta-Amylase mg. maltose/min./g.	Alpha-Amylase (Relative Effect on Viscosity)
Coarse bran	192	7.2
Germ	159	5.7
Reduction 1	280	2.2
Reduction 2	307	3.3
Reduction 3	232	9.9

[a]See ref. 174.

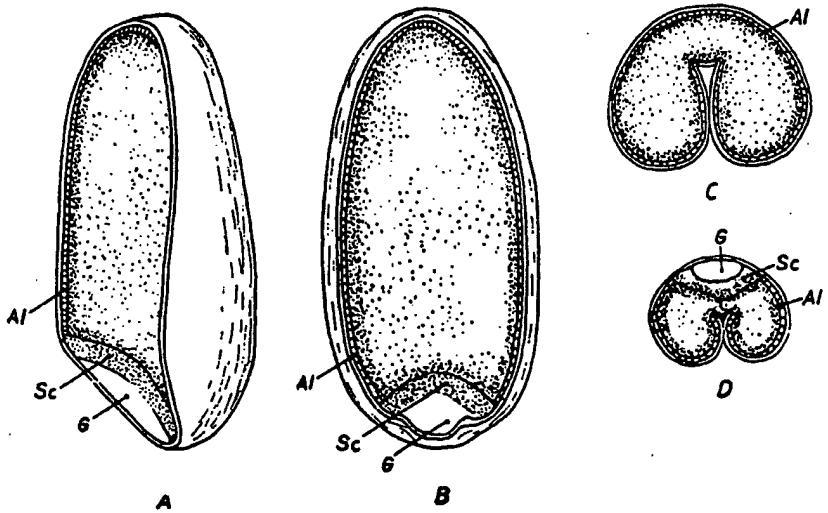


Figure 2. Distribution of saccharogenic amylase in the wheat grain. A, longitudinal section along the crease; B, longitudinal section perpendicular to the crease; C and D, transverse sections of the grain; G, germ; Sc, scutellum; and Al, aleurone layer. Enzyme activity increases with increase in dot intensity (ref. 38).

TABLE VI

Effect of variety and germination of cereals on total saccharifying activity[a]

Cereal		Ungerminated[b]		Germinated[b]	
Species	Variety	Total	Free	Total	Free
Barley	Wisconsin Winter	77.2	43.0	92.9	83.0
	Tennessee Winter	71.1	40.1	80.6	70.2
	Tennessee Beardless	49.6	20.1	58.8	48.1
	Esaw	59.9	32.8	...	...
Wheat	Federation	104.1	55.5	132.4	119.8
	Marquis	116.2	59.2	170.9	138.9
	Baart	110.0	52.9	144.9	104.7
	Golden	90.1	44.8	93.0	77.8
Rye	Dakold	93.4	71.9	137.9	129.0
	Star	89.3	70.9	122.0	118.3
	Rosen	110.5	89.7	127.4	105.8
	Abruzzi	85.0	65.8	104.2	99.0
Oats[c]	Fulghum	3.7	4.5	24.2	25.6
	Iogold	1.5	1.5	22.6	25.6
	Lee	1.5	2.2	16.6	18.1
	Winter Turf	0.7	0.7	16.6	18.1

[a]See ref. 33. [b]Activity is expressed in °Lintner.

[c]Small differences between total and free saccharifying activities are not significant.

called "free" amylase. While there are considerable differences between varieties within each cereal, the barleys have somewhat lower activities than the wheats and ryes. The use of barley malt as the principal enzyme source for production of beer is based largely on some physical characteristics such as the adhesion of the hull to the barley kernel after threshing, and on flavor considerations. Where such considerations are not important, for instance in the supplementation of wheat flour with alpha-amylase for bread-baking, other sources of amylase such as wheat malt and fungal amylases can be used. At present, barley-malt flour and wheat-malt flour are the commercial sources of enzymes for incorporation with wheat flour at the mill; and fungal amylases, malt flours, and extracts are used for supplementation in the bakery.

Oats have little or no amylase activity in the dormant stage but develop saccharifying activity on germination. Buckwheat, corn, rice, and sorghum have activities as low as or lower than those shown for oats. Recently, Fleming et al. (47) have determined the alpha-amylase activities of malted wheat, barley, oats, corn, and sorghum.

During maturation of barley the susceptibility of raw starch to alpha-amylase remains constant until the moisture content of the grain is reduced to 60%. Thereafter susceptibility decreases, presumably because of an increase in retrogradation of the starch. During germination, susceptibility of the starch to enzymolysis increases again. For Seneca wheat digestibility increased from 34% in the original seed to 48% in the wheat malt, and for Butler wheat the increase was of similar magnitude (118).

E. Properties of Wheat Alpha- and Beta-Amylase

EFFECT OF pH

The determination of a so-called pH optimum is usually carried out by plotting the pH value of a well-buffered substrate vs. enzyme activity at a given enzyme and substrate concentration and at a given temperature. Actually the nature of the buffer, the concentration of enzyme, and the temperature affect the pH optimum, so that the value for pH optimum constitutes an approximation which is generally correct only within a tenfold range of hydrogen-ion concentrations. The pH optimum for barley alpha-amylase is about 4.5 and that for wheat alpha-amylase is similar. Below pH 4, activity drops off rapidly and above pH 5, more slowly.

In Northern Europe incipient sprouting of wheat is a constant danger. In Sweden determination of the falling point number (alpha-amylase action) is used for classification and pricing of wheat and rye flours. The falling number is lowest at pH values between 5.2 and 5.7 (meaning that enzyme activity is highest), and acidification of doughs to pH values of 4 or below has been recommended to reduce alpha-amylase activity (131).

The pH optimum for beta-amylase is slightly higher than that for alpha-amylase. At higher temperatures the optimum pH is shifted toward more alkaline values. At 40° and 50°C. the pH optimum was at 4.6; and at 60°C. it was at 5.5 (157,158). It is likely that this shift reflects the higher stability of wheat beta-amylase at the higher pH values (122).

For three fractions of the wheat beta-amylase system which differed in their

Cereal Science & Technology
Edited by G.H. Palmer
Aberdeen Univ. Press 1989

SECTION III

A: THE COVERING LAYERS AND ALEURONE LAYER

A.1: THE HUSK

The husk (glumes) are leaf-like structures which enclose the developing barley grain (Figs 1, 18, 30, 35, 39a,b). The upper dorsal half, which covers the embryo, is called the lemma; the lower ventral half is called the palea. The lemma usually extends into a serated awn at the distal (non-embryo) end of the grain (Fig. 1). Although husk weight can vary, it is about 10 per cent of the dry weight of the grain and is held to the underlying pericarp by cuticular material (Freemen and Palmer, 1984a). However, some barleys, like Himalaya and Nacta are huskless, because like wheat, rye, sorghum, rice and millet grains, the husk is normally detached from the grain during threshing. The lemma and palea usually overlap. In some barley varieties they do not. This feature of the grain can be used to differentiate between varieties and may occur in samples which show a tendency to split.

In commercial practice, husk loss can occur as a result of mechanical damage which takes place during (incorrect) combining and conveying. The silica content of the husk is reported to be high (Kent, 1983) and contributes to its abrasive qualities. Since the pentosan content of the dehusked grain is about 4 per cent (Table 11), and since the pentosan content of the husk-covered grain is about 9.0 per cent, the husk must contain significant quantities of pentosan. In contrast, β-D-glucan results in this Table indicate that the β-D-glucan content of the husk (and pericarp) of the mature grain is negligible. The husk also contains cellulose and lignin. Figure 35 shows that the outer epidermal cells of the husk are cuticularised (see Freeman and Palmer, 1984a). Below the epidermis are thick-walled cells which give the husk its rigidity and strength. The inner husk consists of thin walled cells which adjoin the cementing (cuticular) material on the surface of the pericarp (Fig. 18). Grain impaction damages the inner husk, causing husk loss and reduces the quality of the grain for malting and brewing. Germination of partly-dehusked (skinned) samples of grains will be uneven, resulting in uneven enzyme development and uneven modification, which can cause brewhouse problems (van Eerde, 1983; Aalbers and van Eerde, 1986). The husk is an important component of the coarse fraction of the grist (milled malt). Excessive husk damage can impede wort filtration from the mash tun. The husk, like the underlying pericarp of mature barley grains usually contain tolerable levels of fungi and bacteria (Flannigan and Healy, 1983). As stated above, delays in the harvesting of higher moisture barley (> 16%) can lead to excessive development of field fungi such as *Alternaria*, *Cladosporium* and *Fusarium*. Storage of high moisture grain can lead to unacceptable development of storage fungi such as *Penicillium* and *Aspergillus*. Weather stained (weathered) barley is often associated with fungal activity and, in some quarters, is rejected as malting barley, even though it germinates satisfactorily.

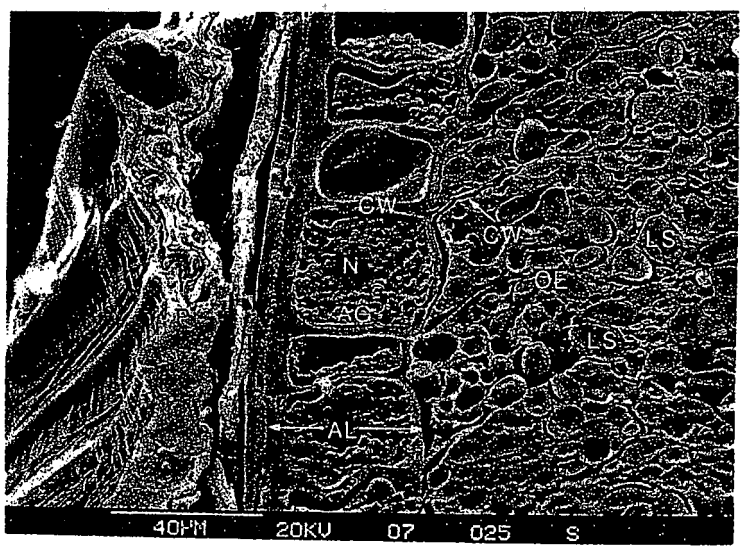


Figure 17: Shows pericarp, testa, aleurone and adjoining outer area of starchy endosperm (soft wheat). P = Pericarp; T = Testa; AL = Aleurone layer (1 cell deep); N = Nucleus; AG = Aleurone grains; OE = Outer endosperm.

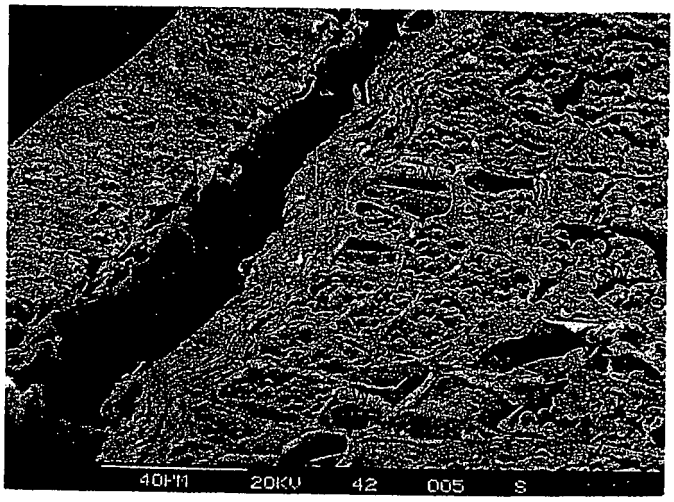


Figure 18: Shows husk, pericarp, testa, aleurone layer and outer areas of the starchy endosperm (barley). H = Husk (partly separated from pericarp); P = Pericarp; T = Testa; AL = Aleurone layer (3 cells deep). Aleurone cells containing aleurone grains; CW = Cell wall; PS = Protein matrix and small starch granules.

Some species of *Fusaria* have been linked with mycotoxin production (Flannigan, 1986) and with beer gushing (Hackara, 1983). Fungal infection of cereals intended for foods can be dangerous to the health of human and animals. *Thermoactinomyces vulgaris* is one of the causative agents of the respiratory disease called farmers's lung. *Aspergillus clavatus* is a toxigenic fungus. The spores, if inhaled, can produce mycotoxins and can cause allergic respiratory disease (allergic alveolitis), better known as malt-worker's lung (Flannigan 1986). *Aspergillus fumigatus* can cause a similar allergic response. Excessive fungal infection of the husk can impair germination by inducing the condition called water sensitivity (Major and Roberts, 1968).

The husk has various morphological features such as pigmentation, pale-alemma structure, shape of point of attachment (bevel/nick), coarseness and thickness. These can be used to assist in the identification of barley varieties (*Pauls Brewing Room Book* 1986–1988). The husk contains a wide range of phenolic substances, some of which are leached out during steeping (Eastmond and Gardnier 1974; Briggs *et al.*, 1981). Incrementally steeped barley, in which added water is absorbed by the grain, can produce beer which is extremely astringent. This astringency is associated with phenolic substances which are leached from the husk during conventional steeping. The husk absorbed water rapidly during steeping but lost it quickly in conditions of reduced humidity (Table 15; Palmer, 1967). During malting, the restriction of moisture loss from the husk by the maintenance of high relative humidity (98–100%) especially during the first three days of malting, serves to maintain critical water relations of the embryo, the aleurone and the starchy endosperm during enzymic modification.

In some breweries, fragmentation of the husk during milling is reduced by steam conditioning, rapid steeping or fine spraying. Such treatments may increase the moisture content of the malt from 5 to 20–25 per cent. The highly absorbent properties of the husk means that, at this malt moisture, the husk could contain, at least, 50 per cent moisture (Table 15; Section IX A).

A.2: THE PERICARP

The husk is a parental leaf tissue; in contrast, the pericarp is maternal tissue of the developing grain and is derived from the ovary wall (Fig. 1). Fruits contain testa and pericarp layers. Seeds contain testa layers—they do not have pericarp layers. Cereal grains contain pericarp layers and are therefore dry (indehiscent) fruits, called caryopses. The pericarp is composed of an outer epidermal layer of cuticular material to which the inner husk is attached (Figs 17, 18, 35). Below the cuticular material are, the main body of crushed 'woody' cell wall material and inner layers of tube and cross cells (Freeman and Palmer, 1984a). The cross cells (bees wing in appearance) are usually one layer thick in wheat, rye, rice and oats but are two or three cells thick in barley and, in the developing grain, are reported to have photosynthetic properties (Duffus, 1985). Unlike the pericarp of sorghum, barley pericarp does not contain starch. In wheat and sorghum grains the shoot (acrospire) breaks through the pericarp-testa after germination is completed. In normal (husked) barley, the acrospire lifts the pericarp-testa as it forces its way, fully protected, over the dorsal surface of the grain (Figs 4, 29, 39a,b 42a, 45), before emerging at the distal end of the grain.

The pericarp of barley is impervious, not only to carbon dioxide (Radley, 1979a), but to gibberellic acid, except at the micropyler (embryo) end of the grain (Freeman and Palmer, 1984a,b). Therefore in undamaged (unabraded) grains exogenously applied gibberellic acid enters the aleurone after absorption by the roots and is transported through the embryo (Figs 30, 31). The permeability of the pericarp to gibberellic acid changes during grain development. During the early stages (20–25 days post anthesis) the pericarp was permeable; in the later stages (36–52 days post anthesis) it was not, even though the aleurone was active from 20 days post anthesis (Freeman and Palmer, 1984 a,b; Cornford *et al.*, 1986).

Wellington reported (1966) that grains with green, inner pericarps germinated badly. Palmer (unpublished) observed that samples of Triumph barleys, which had thickened areas in the cell walls of the inner pericarp cells, germinated slower than those which had thinner walls. Damaging the pericarp not only accelerated germination (Pollock, 1962) it also became a direct portal through which exogenously applied gibberellic acid entered the underlying aleurone layer, thereby optimising the gibberellic acid—aleurone response in malting barley (Palmer, 1969a,b). This process of controlled pericarp damage is called abrasion (see Section V B). Dehusking of barley using cold 50 per cent sulphuric acid removes the husk, and most, if not all, of the pericarp (Fig. 29). Wheat brans contain mainly pericarp-testa and aleurone (Fig. 17).

A.3: TESTA-NUCELLUS OR THE TESTA

The lipid layers of the testa-nucellus, usually called the testa, are located between the pericarp and the aleurone layer (Figs 1, 35). The testa lies below the pericarp and the nucellus lies below the testa. The testa, nucellus and the pericarp are diploid, maternal, tissues of the ovary of the developing grain. The nucellus is absorbed by the developing (triploid) starchy endosperm, leaving a cuticle which persists as the inner lipid layer of the testa of the mature grain (Fig. 35). The testa of the mature grain is usually represented as two lipid (cuticular) layers. Cellular materials can sometimes be found between these lipid layers (Freeman and Palmer (1984a)). Testa tissue is less compressed over the embryo than over the endosperm (Fig. 29) and is highly resistant to cold 50 per cent sulphuric acid, which is normally used to 'dehusk' barley (Freeman and Palmer, 1984a,b). Palmer (1969) reported that the testa of sulphuric acid dehusked grains was permeable to gibberellic acid. Smith and Briggs (1979) failed to observe this. However, testa permeability was confirmed later by Briggs and MacDonald (1983a). The different results from Briggs' laboratory related more to difficulties with techniques rather than variability of the response of the testa to gibberellic acid. Freeman and Palmer

(1984b) found that radioactive gibberellic acid failed to penetrate the pericarp but readily permeated the testa.

Structurally, the pericarp forms a complete layer over the endosperm. The testa runs into the chalazal area of the furrow (or crease), and is incomplete (Freeman and Palmer, 1984a). Collins (1918) reported that the testa is semi-permeable, allowing access to some substances while excluding others from reaching the aleurone layer. Radley (1979a) has attested further to the impervious nature of the pericarp by reporting that the pericarp, rather than the testa, limited the ingress of carbon dioxide into the grain. The testa layers of some Sudanese sorghum varieties contain pink-red pigments and, Aastrup *et al* (1984) have reported that proanthocynidin pigments in barley are present in the testa.

A.4: THE ALEURONE

As stated above (Section I B,C) the aleurone layer (~ 10% grain weight) and the starchy endosperm are derived from the same triploid nucleus after fertilisation. During development of the grain, the inner mass of endosperm cells synthesise starch and protein, while the outermost cells differentiate into (living) aleurone cells which contain lipids (~ 30%), proteins (~ 20%), phytic acid, B-Group vitamins, pentosan-cellulose, minerals and sucrose but are free of starch (see Palmer and Bathgate, 1976; Charalambous and Bruckner, 1977; Kent, 1983; Palmer, 1987a). The aleurone layers of malting barley are usually three cells deep (Figs 18, 29a, 35, 43). However, aleurone tissue containing one layer of cells is sometimes found, especially near the scutellum of the embryo (Figs 29a, 43). In contrast to barley, the aleurone layers of wheat (Fig. 17), oats, rye, sorghum (Fig. 68), millet, maize, rice and triticale are usually one cell layer in thickness. The aleurone layer of Galant barley was observed to be three to four cells deep (Palmer, 1988).

Table 9 shows that the cell walls of the aleurone layers of wheat or barley contain similar levels of arabinoxylans (65–67%) and β-D-glucans (29%–26%); see Fincher and Stone (1986). Although pentosans and β-D-glucans are the major chemical substances of the aleurone cell wall, small quantities of protein, cellulose, glucomannan and phenolic compounds are also present. The aleurone cell walls are traversed by plasmodesmata (Paleg and Hyde 1964), which, like those of the starchy endosperm cell walls (Palmer, 1987b), are composed of phospholipids. The aleurone, unlike the starchy endosperm, is living tissue. Protein, phytic acid and carbohydrate materials are deposited in subcellular organelles called aleurone grains (see Figs 17, 18; Jacobson *et al.*, 1971). Aleurone layers are major components of bran, contributing cell wall fibre and limited quantities of lipid, protein, minerals and vitamins (Table 28). Recent studies by Palmer (1987a) showed that whereas high levels of phosphorus (phosphate) and potassium were present in the aleurone layers of barley, wheat, rice, oats, triticale and rye, significantly lower levels were detected in the aleurone layers of maize, sorghum and millet, suggesting that the nutritional value of the aleurone may vary between different cereals

(Palmer, 1987a). It is not clear whether or not differences in the chemical composition of the aleurone grain can affect the potential of the aleurone layer to produce endosperm degrading enzymes. However, it was observed that gibberellic acid failed to induce α-amylase synthesis in those cereals which had low phosphorus (phosphate) content (Palmer, 1987b).

Paleg, 1960a,b; MacLeod *et al.*, 1964; Varner and Ram-Chandra, 1964; Palmer, 1970 reported that the aleurone layer produced and secreted endosperm-degrading enzymes. MacLeod and Palmer (1966); Palmer (1967; 1969; 1980b; 1982) reported that, in malting grain, the aleurone initiated endosperm breakdown and that the potential of excised barley embryos to produce α-amylase reflected contamination of the scutellum with aleurone cells (MacLeod and Palmer, 1966; Palmer, 1967; Smart and O'Brien, 1979 and Palmer, 1982). α-Amylase and protease are produced, *de novo*, in the aleurone, as new protein molecules (Varner and Ram-Chandra, 1964; Varner, 1978; Jacobsen *et al.*, 1979). The cytoplasm of the aleurone of ungerminated barley contains endoplasmic reticulum, ribosomes, microbodies, dityosomes and plasmolemma. These subcellular structures are more prominent when the aleurone is hydrated and stimulated by gibberellic acid. The polysomal nature of the endoplasmic reticulum is indicative of its potential to synthesise endosperm-degrading enzymes. Jones (1969) suggested that endosperm-degrading enzymes (*eg* α-amylase) were released into the cytoplasm and transported freely out of the aleurone cells. Gibson and Paleg (1976) indicated that subcellular transport was particulate. Varner (1978) reported that in the aleurone, oxygen was required for both the synthesis and secretion of endosperm-degrading enzymes.

Paleg (1960a,b) established that gibberellic acid induced excised endosperms to breakdown their own endosperms, releasing reducing sugars. MacLeod and Miller (1962); MacLeod *et al* (1964) showed that gibberellic acid-treated aleurone layer produced and released α-amylase, endo-β-glucanases, proteases and pentosanases (Table 14, Figs 25, 26, 26a). Varner and Ram-Chandra (1964) reported that α-amylase synthesis was associated with a small fraction of the messenger—RNA (Ribose Nucleic Acid) found in gibberellic acid-treated aleurone layers. This small fraction of gibberellic acid-induced m-RNA was polyadenylated, *ie*, contained poly (Adenosine) residues (Jacobsen *et al.*, 1979). Higgins *et al* (1976) isolated this poly (A) RNA and, using the translation ribosome system of wheat germ, found that it induced α-amylase synthesis *in vitro*. The corresponding m-RNA from the water-treated aleurone of barley did not produce α-amylase *in vitro*. Although Filner and Varner (1967) suggested that, like α-amylase, endo-protease enzymes are synthesised *de novo* in the aleurone, there is no corresponding evidence that all the other aleurone-produced enzymes (Table 14), which require gibberellic acid for development are synthesised *de novo* (Tables 15a; 15b). Recent work (Palmer, 1988) which showed that the response of the pro-anthocyanidin-free aleurone, of Galant barley, to gibberellic acid was poorer for endo-β-1,3; 1,4-glucanase than for α-amylase implies that the mechanism of production of these two enzymes may be different.

ENZYMES
and Their Role in Cereal Technology

Edited by

James E. Kruger
Canadian Grain Commission
Winnipeg, Manitoba, Canada

David Lineback
North Carolina State University
Raleigh, North Carolina, USA

Clyde E. Stauffer
Technical Foods Consultants
Cincinnati, Ohio, USA

Published by the
American Association of Cereal Chemists, Inc.
St. Paul, Minnesota, USA

1987

inverse correlation has been reported between seed abscisic acid content and germination (King, 1976; Slominski et al, 1979). However, such correlations are not always observed (King, 1982). A relationship has been observed between onset of dehydration, gibberellin-induced amylase production, and the decline of abscisic acid content in a range of cereals (King, 1982). Such experiments do not show that abscisic acid is solely responsible; indeed, many other factors are involved in the control of premature germination (Cameron-Mills and Duffus, 1977).

The auxin of immature cereal endosperms may be derived from the cytoplasm of the disintegrating cells adjacent to the embryo (Hatcher, 1943). Its role, if any, in grain development is unknown.

VI. THE MATURE SEED

The later stages of maturation are characterized by a steady process of dehydration accompanied by a slowing down of enzymatic and assimilatory activity. The seeds are then harvested and stored until required. Figure 6 is a diagram of a barley grain in longitudinal section. The moisture content of mature seeds is a key to their metabolic activity and should ideally have fallen to 14% or less. Above 14% moisture content, respiratory processes can become significant and deterioration by microorganisms rapid. Temperature obviously affects these processes also.

Estimating the metabolic activity of the dry resting or quiescent seed is difficult largely because most experimental techniques involve the addition of water to provide a medium for following enzyme-catalyzed reactions. This of course would (in the absence of dormancy) trigger the metabolic processes associated with germination. Evidence of oxygen uptake and carbon dioxide evolution does not necessarily mean that respiration is taking place. For

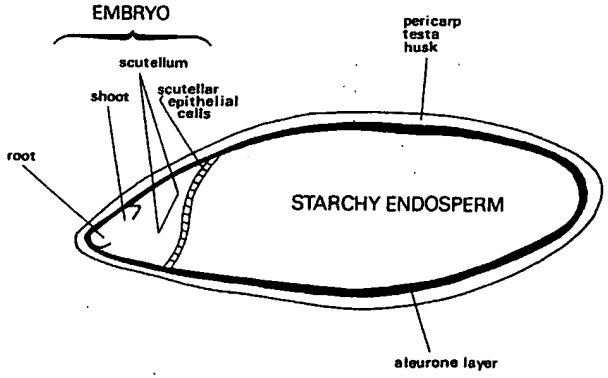


Fig. 6. Longitudinal section of a barley grain.

Ponpipom, 1977), but it is difficult to generalize because both surface erosion and channeling and interior hydrolysis occur.

DEGRADATION OF STARCH DURING END-PRODUCT PROCESSING

This subject is covered in depth in chapters dealing with the end-product processes associated with the different cereals. Note, however, that the properties exhibited in dilute solution of a purified carbohydrase may not always be the same as in an end-product process, such as breadmaking, where water is limited and protection is offered by substrate. For example, the "green" α-amylase group in germinated wheat is more thermolabile than the "germinated" group (Kruger and Marchylo, 1985). Both groups had identical effects, however, in lowering amylograph viscosities, indicating that the high starch-water ratio must be conferring added stability to the "green" group.

III. β-AMYLASES

Cereal β-amylases cleave alternate α-(1→4) D-glucosidic linkages in starch components in a stepwise fashion from the nonreducing end, resulting in the production of β-maltose. The action of the enzyme stops in the region of α-(1→6) D-glucosidic linkages. Complete degradation of linear dextrin chains containing an even number of D-glucose residues results in the production of maltose, whereas those with an odd number result in the production of maltose and a single D-glucose residue. Degradation of branched starch molecules, such as amylopectin, results in the formation of maltose and a β-limit dextrin.

A. Methods of Determination

β-Amylase is difficult to determine in cereals because of the interference normally arising from the presence of α-amylase. This complication may be less important in end-product processing, where measurement of the combined effect of the two enzymes may be desirable. For example, in breadmaking, it is necessary to know that both enzymes are present at the required levels to ensure that sufficient sugars will be produced during the fermentation period. In general, this is accomplished by measuring the amount of reducing sugars liberated by autolysis of the flour sample being used. For example, the maltose value (AACC, 1983, Method 22-15) is a test in which 5 g of flour is mixed with 4 ml of buffer at pH 4.7 for 1 hr at 30° C and the reducing sugars in the filtrate are determined by the ferricyanide method. Another commonly used method is the determination of the gassing power (AACC, 1983, Method 22-11) in which gas production is measured after 6 hr in a fermenting dough. The test suffers from the complication that it is strongly influenced by the amount of damaged starch granules in the flour.

A number of the reducing sugar methods for measuring β-amylase use soluble starch as substrate, particularly in assessing the amount of the enzyme in barley and malt. The methods for measuring the reducing sugars vary and may involve reduction with 3,5-dinitrosalicylic acid (Bernfeld, 1955) or neocuproine (Bureau and MacGregor, 1980) to form a colored complex. Occasionally some treatment, such as performing the test at pH 3.4 in order to inactivate α-amylase, is employed (Warchalewski and Tkachuk, 1978). Such attempts to preferentially inactivate α- or β-amylase are generally not completely satisfactory because the treatment may partially inactivate the desired enzyme. Recently, Mathewson and Seabourn (1983) have described a new method employing *p*-nitrophenyl oligosaccharides which specifically measures β-amylase in cereals in the presence of α-amylase.

Note that β-amylase is partially bound in cereals. To determine the amount of this "bound" enzyme, in addition to buffer-soluble or "free" enzyme, a reductant, such as cysteine, or a proteolytic enzyme, such as papain, must be added.

B. Anatomic Distribution and Amounts in Cereals

During wheat kernel development, the amount of β-amylase in the endosperm increases (Schwimmer, 1947; Kruger, 1972). As final maturation approaches, however, the enzyme becomes increasingly bound, such that only about 20% is soluble. This bound enzyme is attached to the glutenin proteins via disulfide bonds (Rowsell and Goad, 1962a, 1962b; Kruger, 1970). The pericarp also contains a β-amylase component which increases during early kernel development along with pericarp α-amylase (Kruger, 1972). This enzyme disappears at the later stages of kernel development, in contrast to the endosperm β-amylases. Its role may be to facilitate the early breakdown of starch in the pericarp.

Upon germination of wheat and barley, the amount of β-amylase in the endosperm increases several fold, not because of de novo synthesis of new enzyme, but mainly because of the proteolytic or disulfide reductase enzyme-mediated release of the bound β-amylase. Okamoto and Akazawa (1980) found, however, that part of the β-amylase of rice is synthesized de novo in the scutellum during the early stages of germination. At the later stages, inactive, latent β-amylase components that were associated with the starch granules in the endosperm were activated, and the amounts of these components finally predominated.

C. Multiple Forms of β-Amylase

The occurrence and nature of multiple forms of β-amylase are much more complicated than for α-amylase. Not only do a number of forms result from genetic variation, but also the multiple forms may aggregate by disulfide bonds, either with themselves or with other proteins (Nummi et al, 1965, 1972).

Tkachuk and Tipples (1966) first noted the existence of three major and two minor β-amylase isoenzymes in mature wheat using ion-exchange chromatography. Kruger (1972), using gel electrophoresis, found that two main components were present in the endosperm and that these slowly increased throughout development. At the later stages of development, small amounts of four electrophoretically slower components gradually appeared. β-Amylase was also present in the pericarp and consisted of one form. The "free" and "bound" β-amylases present in the endosperm had identical electrophoretic abilities. Two main forms were also found using ion-exchange chromatography (Kruger, 1970), with the free and bound forms again appearing identical. Using isoelectric focusing, Kruger (1979) established that one of the components was

isoenzymes present. This phenomenon may be caused by kernel shriveling and the onset of the germination process. However, Peña and Bates (1982) and Peña et al (1982) have reported that α-amylase activity does not affect kernel shriveling in triticale.

C. Anatomic Distribution and Amounts in Cereals

The amount of α-amylase in cereals depends on the natural state of the caryopsis, i.e., whether it is immature, resting, or germinated. In wheat and barley, α-amylase can be detected shortly after flowering; amounts increase

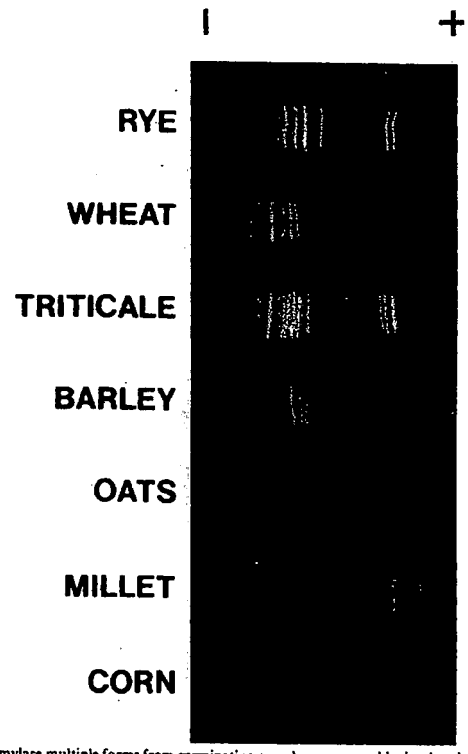


Fig. 1. α-Amylase multiple forms from germinating cereals as separated by isoelectric focusing on a polyacrylamide gel.

initially, then decrease with further maturation and desiccation. The residual content depends on the cultivar. In some cases, cereals may have elevated concentrations of α-amylase in the resting state following a wet harvest season without any external evidence of germination. This is referred to as incipient sprouting. Upon germination of the grain, the concentrations of the enzyme increase several hundred fold over a 4- or 5-day period.

α-Amylase in immature wheat and barley kernels is predominantly located in the pericarp, with small amounts present in the seed coat and aleurone. In germinated wheat and barley, the anatomic location of the enzyme depends on the degree of germination. Although the enzyme is known to be synthesized de novo, the actual sites of early synthesis have been the subject of considerable controversy, particularly with regard to the respective roles that the scutellum and the aleurone tissues play in this process (see Chapter 4). However, the enzyme initially appears next to the distal side of the endosperm adjacent to the scutellum and aleurone layer. With increasing germination, the enzyme progressively penetrates the aleurone and gradually moves toward the nondistal end of the caryopsis.

The total amount of α-amylase and its location in the caryopses of germinated or malted kernels have important implications for technological processes. In barley, it is desirable to have the starch in the malted kernels completely modified or broken down into simple compounds for subsequent fermentation. Thus, at the end of the malting process, low amounts and incomplete penetration of enzyme lead to "undermodified" malts of poor quality. In wheat, on the other hand, low amounts of the enzyme are desirable. The extent of penetration into the endosperm, which is reflected by the severity of sprouting, has practical quality implications. Thus, the actual distribution of α-amylase in the mill fractions themselves can be affected (Kruger, 1982). In practice, removal of the later reduction flour streams to minimize the α-amylase content is not done, presumably because it is not economically feasible. Another consequence of differences in the severity of sprouting is that, because the amount of enzyme in the endosperm varies relative to that in the entire kernel, the milling process easily removes at least 50–60% of the α-amylase (Kruger and Tipples, 1980). This affects the relationship between the Hagberg falling number method for determining α-amylase using ground wheat and the amylograph method using flour.

D. Chemical and Physical Properties

The α-amylases present in cereals have broadly similar properties but are quite different from α-amylases from fungal or bacterial sources. These differences are very important in technological processes such as breadmaking. For example, thermal stabilities of α-amylases from cereals are greater than those of α-amylases from fungal sources but less than those of α-amylases from bacterial sources. Thus, whereas fungal or cereal α-amylase may be used as a supplement to enhance and sustain gas production, use of bacterial α-amylase can lead to excess dextrinization during the baking process, because of its high thermal stability, and a deterioration of bread quality.

Although broadly similar, cereal α-amylases, and even individual isoenzymes of a particular cereal, do differ. Some properties for which comparisons can b